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SHORT COMMUNICATION

Early fertilization events in the sexual and aposporous egg apparatus of *Pennisetum ciliare* (L.) Link

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Summary

While detailed descriptions exist on the fine structural characteristics of double fertilization in sexual angiosperms, few studies have described fertilization in apomictic species. The results of this study with buffelgrass (*Pennisetum ciliare* (L.) Link syn= *Cenchrus ciliaris* L.) show that differences exist between the early fertilization events in sexual and apomictic (aposporous) female gametophytes. In unpollinated sexual female gametophytes, the egg cell did not show signs of parthenogenetic activity. In contrast, autonomous egg embryony was observed in unpollinated aposporous female gametophytes. Compared with the sexual genotype, degeneration of the synergids in the aposporous female gametophyte was precocious and rapid. During the first 4 h after pollination, the chalazal end of the sexually functional egg cell remained covered by the plasma membrane. In the aposporous embryo sac, a cell wall covered the plasma membrane of the egg cell several hours before penetration of the pollen tube into the female gametophyte.

Introduction

Few studies have characterized the structure of female gametophytes (embryo sacs) in apomictic plants. Apomixis is a method of asexual plant reproduction through seeds that culminates in the parthenogenetic development of the egg cell, and the formation of viable embryos (Bashaw and Hanna, 1990). Because obligate apomictic plants produce uniform progeny, the genetic transfer of apomixis to cultivated crops has been perceived as an important technological challenge faced by modern plant breeding (Dickinson, 1992). To answer some of the questions related to the mechanisms that regulate female gametophyte development in apomictic species, we have initiated a series of studies that take advantage of the reproductive diversity present within the grasses.

Although two apomictic mechanisms, apospory and diplospory, have been reported in the Poaceae (Asker and Jerling, 1992), apospory is the most prevalent. In most aposporous ovules the meiotically derived megaspores abort, and the differentiation of a somatic unreduced nucellar cell gives rise to a four-nucleated embryo sac (Gustafsson, 1947) which contains the equivalent of two synergids, an egg cell and a polar nucleus (Nogler, 1984; Figure 1). While this pattern of differentiation may vary, antipodals normally are not present at the chalazal end of the central cell. In most cases, the fusion of sperm and egg cell is prevented, but fertilization of the polar nucleus(ei) is often required for the production of viable seed.

Although obligate apomictic genotypes produce progeny genetically identical to the maternal plant, most aposporous species reproduce by facultative apomixis (Harlan and de Wet, 1963). In facultative apomictic genotypes, sexual and apomictic embryo sacs coexist within the same ovule (Figure 1). Since the mechanisms which regulate the development of sexual and apomictic embryo sacs are not understood and are influenced by several environmental conditions (Hussey *et al.*, 1991; Knox, 1967), facultative apomictic plants make poor experimental material for detailed comparative studies of apomixis.

Buffelgrass (*Pennisetum ciliare* (L.) Link syn= *Cenchrus ciliaris* L.) is a drought-tolerant grass which is widely used as a forage species throughout the semi-arid tropics (Bogdan, 1977). In contrast to most aposporous species where facultative apomictic genotypes are prevalent, many genotypes of *P. ciliare* reproduce by obligate apomixis. The discovery of a sexual plant of *P. ciliare* (Taliaferro and Bashaw, 1966) gave rise to obligate sexual genotypes that only produce reduced female gametophytes of the Polygonum type (two synergids, an egg cell, two polar nuclei, and three or more antipodals; Figure 1). In addition, hybridization of sexual and aposporous genotypes produces hybrid populations (F_1) which contain both aposporous and sexual plants (Bashaw and Hignight, 1990).

This paper presents a comparison of the sexual and aposporous egg apparatus (synergids and egg cell) in unpollinated and pollinated pistils prior to the pollen tube entry into the female gametophyte. Following criteria suggested by Sherwood *et al.* (1980), we selected an obligate

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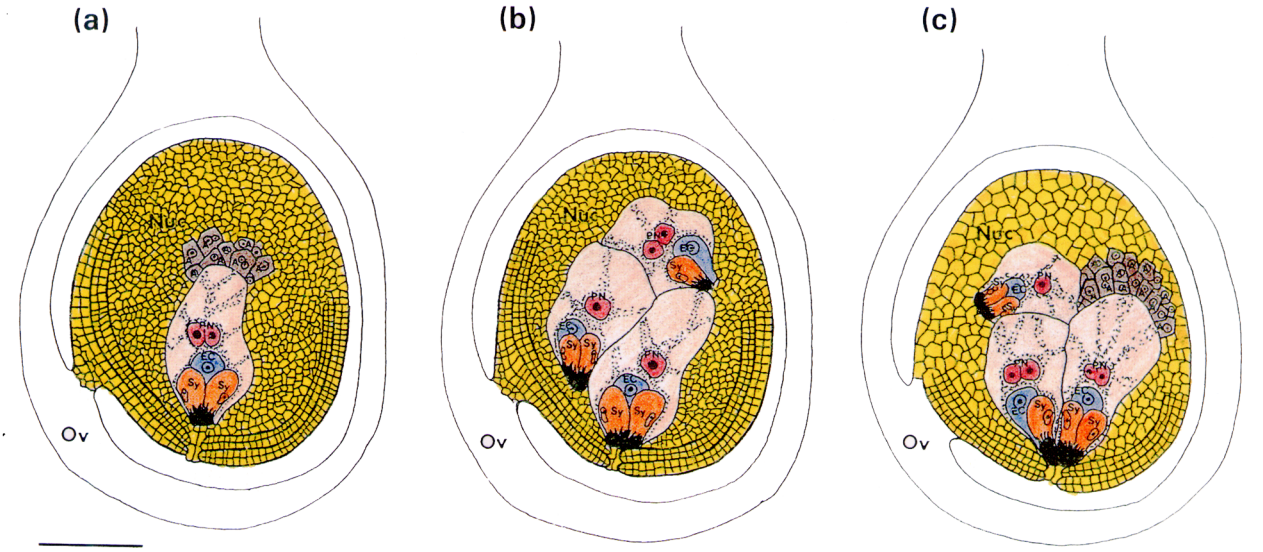

Figure 1. Method of reproduction and organization within the ovule of a typical aposporous apomictic species.
In obligate sexual genotypes (a) a single female gametophyte of the Polygonum type is present within the ovule. In obligate apomictic genotypes (b) one or several aposporous female gametophytes may be present within the ovule. In facultative apomictic genotypes (c) sexual and aposporous female gametophytes coexist within the ovule.
A, antipodals (brown); EC, egg cell (blue); Nuc, nucellus (yellow); Ov, ovary (white); PN, polar nucleus(ei) (red); Sy, synergid (orange); bar = 0.2 mm.

sexual (BBW178) and an obligate aposporous genotype (BBW291) from an F_1 population. To define the reproductive nature of the selected genotypes, and to quantify cytological events at the light microscopy (LM) level, we used a clearing method that allows the observation of large sample sizes (Young *et al.*, 1979). By observing whole-mounted cleared pistils, we collected quantitative data on the number of female gametophytes present in each ovule, the nature of cells within the embryo sac, and the presence or absence of parthenogenetic proembryos in unpollinated pistils of *P. ciliare*. Because the technique lacks cellular and organellar specificity (Mòl *et al.*, 1994), our results were complemented by a fine structural characterization of the early fertilization events in both genotypes, and in a genetically unrelated obligate aposporous genotype (T4464). During ultrastructural comparisons, we analyzed the temporal pattern of synergid degeneration, the organization of the egg cell cytoplasm, and the distribution of the egg cell wall at several sagittal levels.

This study represents a report on the fine structural characteristics of aposporous female gametophytes and their relationship to fertilization in the angiosperms. We also show that the use of full-sib progeny of *P. ciliare* which differ in method of reproduction (sexual or apomictic) represents a valuable system to study female gametophyte development in the grasses, and constitutes an initial step toward a detailed cellular and molecular analysis of apomictic reproduction.

Results

Organization of the egg apparatus in unpollinated pistils

In unpollinated ovules from the sexual genotype (BBW178), a single female gametophyte contained an egg apparatus composed of an egg cell and two synergids (125 out of 156 ovules observed; Table 1). These three cells were attached at the micropylar apex of the embryo sac and were organized in a typical triangular configuration. The central cell contained two closely associated polar nuclei, and the chalazal end of the embryo sac was occupied by a cluster of antipodal cells. In contrast, the unpollinated ovules of the obligate aposporous genotypes (BBW291 and T4464) contained a variable number of embryo sacs in close association. These female gametophytes displayed different patterns of differentiation. In most cases, the embryo sac contained two synergids, a single polar nucleus, and an egg cell (305 out of 374 embryo sacs in BBW291; 196 out of 249 in T4464; Table 1). The second most frequent pattern of differentiation in aposporous female gametophytes was a single synergid, two polar nuclei, and an egg cell (39 out of 374 in BBW291; 43 out of 249 in T4464; Table 1). Female gametophytes with three associated polar nuclei, an egg cell, but no synergids were also observed (18 out of 374 in BBW291; three out of 246 in T4464; Table 1). On two occasions, an embryo sac of BBW291 contained three polar nuclei with no other cell being visible. Antipodal cells were not observed in the

Table 1. General organization of sexual and aposporous female gametophytes in unpollinated ovules of *P. ciliare*

Genotypes	Number of ovules observed	Number of embryo sacs per ovule (mean)	Types of embryo sac[a] (% of the total)	Percent ovules containing a proembryo
BBW178 sexual	156	1	2Sy+1EC+2PN+As (80.1) Non-differentiated (6.6) Aborted (13.4)	0
BBW291 aposporous	117	3.2	2Sy+1EC+1PN (81.5) 1Sy+1EC+2PN (10.4) EC + 3PN (4.8) 3PN (1.7) ND (1.6)	27
T4464 aposporous	131	1.9	2Sy+1EC+1PN (78.7) 1Sy+1EC+2PN (17.3) EC + 3PN (1.2) ND (3)	7

[a] Sy=synergid; EC=egg cell; PN=polar nucleus(ei); As=cluster of antipodals; ND=not determined. Non-differentiated megagametophytes of BBW178 include two-nucleated, four-nucleated and 8-nucleated non-cellularized embryo sacs. Ovules not containing a female gametophyte were classified as aborted.

aposporous female gametophytes. Parthenogenetic development of the egg cell was observed in 7–27% of unpollinated aposporous ovules (Table 1). These proembryos occupied most of the micropylar region of the female gametophyte and were closely associated to one or several polar nuclei (data not shown).

The pattern of synergid degeneration

In the sexual genotype, the mature unpollinated synergids failed to show signs of precocious cytoplasm degeneration (Figure 2a), and the synergids were separated by a wall sharing a common filiform apparatus in the micropylar region. The synergid nucleus contained a single nucleolus, was located in the center or the micropylar region of the cell, and was appressed to an external wall common to the egg cell or the central cell. Two hours after pollination, the structure of the two cells remained similar; however, their cytoplasmic content appeared less dense, and contained a large number of small vacuoles (Figures 2b, c and 3a). Three to four hours after pollination, signs of synergid degeneration were visible in one or both cells in the sexual female gametophyte (Figure 4a), but no evidence of preferential degeneration of the synergids was observed.

In the aposporous genotypes, the unreduced synergids followed a pattern of development similar to the sexual genotype; however, signs of degeneration were observed in most unpollinated female gametophytes of both genotypes. Under LM, degenerated synergids were characterized by a general collapse of their cytoplasmic volume, a dense cytoplasm, and the eventual disappearence of the nucleus (Figure 2d). Under transmission electron microscopy (TEM), the cytoplasm was electron-dense, the plasma membrane appeared disrupted, organelles could not be identified, the nucleus was irregular in shape and progressively became unrecognizable (Figure 3b). Two hours after pollination, the degenerated synergid had collapsed and its remnants appeared as a thin electron-dense fringe wrapped around the egg cell in a tight association (Figure 2e and f).

Fine structure of the egg cell

In the sexual genotype, the egg cell was separated from the synergids by a thin cell wall at the micropylar region (Figure 3a). This cell wall was absent at the chalazal end. Three to four hours after pollination, no changes could be detected in the egg cell wall distribution. The collapsed cytoplasm of a synergid created a hook as part of the degenerated material occupied a thin space between the plasma membranes of the egg and the central cell (Figure 4a). At the chalazal region, a large vacuole occupied most of the egg cell volume and the plasma membrane of the synergids and the egg cell appeared to be in contact (Figure 4b).

At maturity, the aposporous egg cell was characterized by a conspicous, centrally located nucleus that contained a single nucleolus (Figure 2e). Before pollination, the egg cell cytoplasm was separated from the synergids by a cell wall at the micropylar region (Figure 3b). Three to four hours after pollination, striking changes were found in the egg cell. A cell wall without a middle lamella completely covered the plasma membrane of the egg, separating the cell from the degenerated cytoplasm of a tightly wrapped synergid (Figure 4c, d and f); however, no substantial changes in the egg cell size were detected. In BBW291, a cell wall was found in seven of 11 female gametophytes

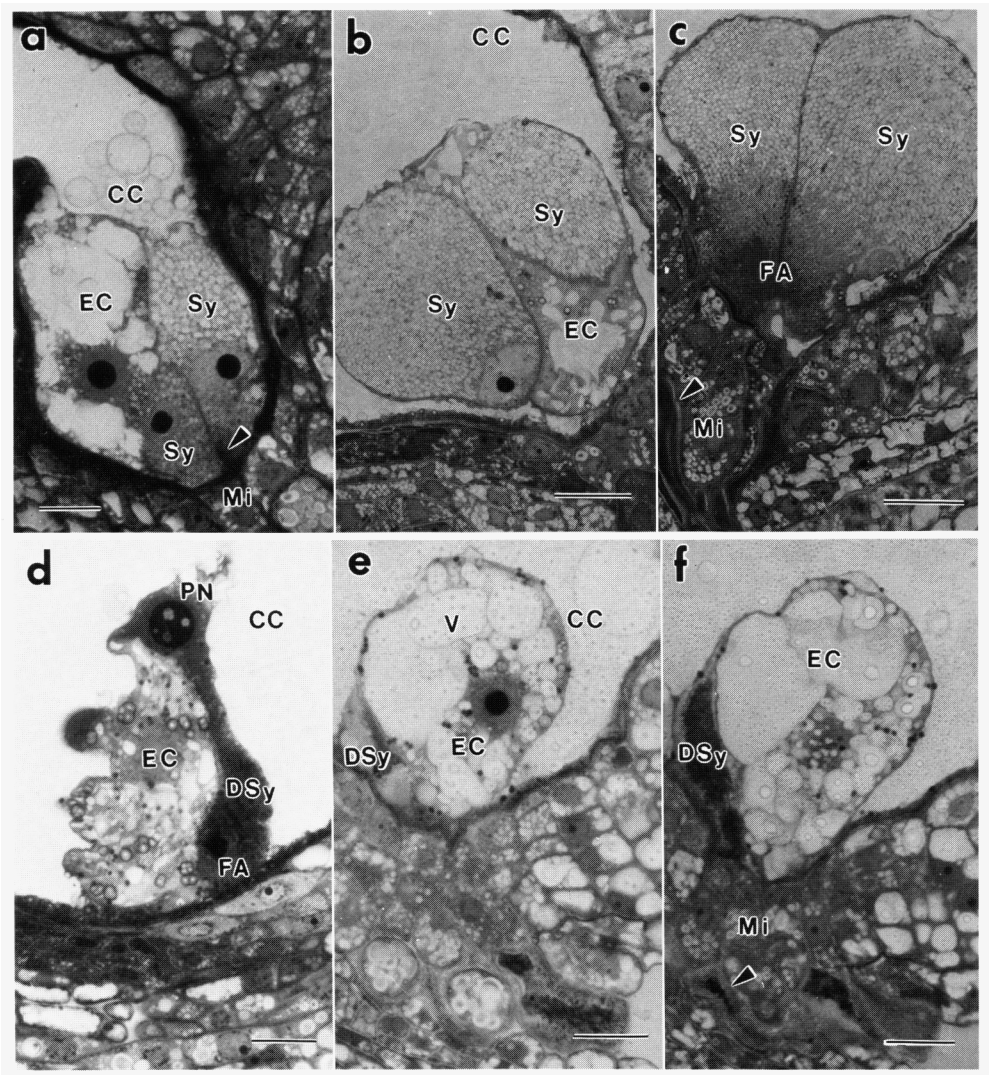

Figure 2. Pattern of synergid degeneration in the egg apparatus of *P. ciliare*; sexual egg apparatus (a–c) and aposporous egg apparatus (d–f).
(a) Sexual egg apparatus prior to pollination. The egg apparatus shows a vacuolated egg cell (EC) and two associated synergids (Sy) sharing a filiform apparatus (arrowhead) at the micropylar end (Mi) of the female gametophyte; bar = 20 μm.
(b and c) Two non-consecutive sections of the same sexual female gametophyte 2 .5 hours after pollination. (b) The synergids (Sy), and the egg cell (EC) are visible; CC, central cell. (c) A pollen tube (arrowhead) has reached the micropyle (Mi) but not the female gametophyte; the synergids (Sy) do not show signs of degeneration; FA, filiform apparatus; bar=20 μm.
(d) Aposporous egg apparatus prior to pollination. A synergid is in a state of degeneration (DSy). The egg cell (EC) and the polar nucleus (PN) are also visible; FA, filiform apparatus; bar=20 μm.
(e and f) Two non-consecutive sections of the same aposporous female gametophyte 2.5 hours after pollination. (e) Egg cell (EC) with centrally located nucleus and vacuole (V); CC, central cell; DSy, degenerated synergid. (f) The pollen tube (arrowhead) has reached the micropyle (Mi) but not the female gametophyte; the cytoplasm of a severely degenerated synergid (DSy) appears closely associated with the egg cell (EC); bar=20 μm.

ultrastructurally analyzed, not including one megagametophyte that contained a parthenogenetic proembryo (data not shown). In T4464, eight of 14 female gametophytes observed contained a complete cell wall. A thin layer of central cell cytoplasm was associated with the external edge of the egg cell wall (Figure 4d and f). The egg cytoplasm appeared vacuolated and contained numerous plastids often organized in clusters at the periphery of the nuclear membrane (Figure 4e).

Discussion

Differentiation of the egg apparatus and degeneration of the synergids

Examination of the sexual unpenetrated egg apparatus of *P. ciliare* illustrates many structural features that have been shown to be conserved in higher plants. The progressive degeneration of one or both synergids during the events

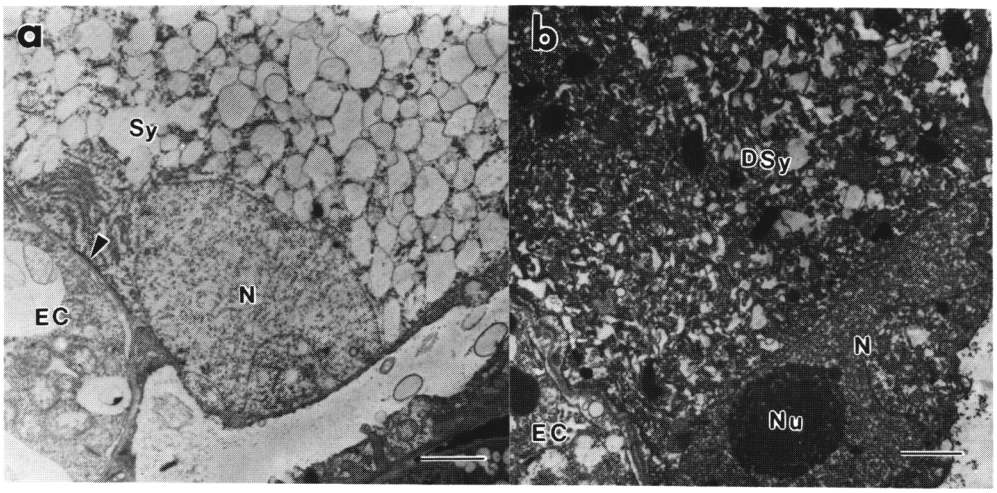

Figure 3. Synergids of *P. ciliare* 2.5 hours after pollination.
(a) Micropylar end of a persistent synergid (Sy) in a sexual female gametophyte. The nucleus (N) appears appressed to the plasma membrane. The cytoplasm is covered by a large number of small vacuoles. A thin cell wall (arrowhead) is visible between the egg cell (EC) and the synergid; bar=1μm.
(b) Micropylar end of a degenerating synergid (DSy) in an aposporous female gametophyte. The synergid cytoplasm, the nucleus (N) and the nucleolus (Nu) are degenerating; EC, egg cell; bar=1μm.

preceding fertilization has been reported extensively (Mogensen, 1988). However, the moment for initiation of the degenerative process seems to vary among species, and the nature of the stimulus for synergid degeneration remains a matter of dispute (Huang and Russell, 1992; Russell, 1992).

Compared with the sexual genotype, the degeneration of the synergids in the aposporous female gametophyte was accelerated. Several hours before the entry of the pollen tube the cells were in a state of degeneration. This observation is supported by the failure to detect preserved synergids in aposporous female gametophytes of whole-mounted cleared ovules. Signs of synergid degeneration in most unpollinated pistils of the two aposporous genotypes indicate that there is variability in the time of initiation of the degenerative process. The differentiation of aposporous female gametophytes raises questions regarding developmental alternatives during female gametogenesis. It also suggests that, on some occasions, differentiation of the synergids may never occur in aposporous female gametophytes of *P. ciliare*.

Activation of the aposporous egg cell

The presence of an incomplete cell wall at the micropylar region of the egg cell has frequently been observed in sexual plants. An inactive state of the egg cell prior to fertilization has also been reported in several species including *Zea* (Diboll, 1968), *Gossypium* (Jensen and Fisher, 1968), and *Capsella* (Schulz and Jensen, 1968). In the absence of ultrastructural studies addressing fertilization events in apomictic female gametophytes, reports on the origin and formation of parthenogenically derived haploid embryos in sexual angiosperms are particularly relevant. In an induced barley mutant, Mogensen (1982) showed that fusion of the plasma membrane of the egg cell and sperm cell was prevented by some physiological means, since no structural explanation could be advanced. Recently, a series of studies on *Ephedra* (Friedman, 1990, 1991) confirmed that the ability of gametes to fuse may be dependent upon the attainment of a precise cell-cycle synchronicity.

Three to four hours after pollination, the aposporous egg cell of *P. ciliare* may have already entered a sporophytic (zygotic) phase that is presumably the result of parthenogenetic activation. Thus, we suggest that the completion of the cell wall may be the consequence of a previous activation process. Based on this hypothesis, several mechanisms may regulate fertilization of the aposporous egg cell. This may be similar to events that occur during microspore embryogenesis in *Brassica*, where the formation of a thick cell wall is the first indication that the cell has entered the sporophytic phase (Zaki and Dickinson, 1990). A precocious cell wall completely covering the egg may constitute a physical barrier that prevents contact between the plasma membranes of the sperm and egg cell. The variable frequencies of proembryos observed in unpollinated ovules suggest that parthenogenesis can be initiated at different times. If activation initiates after pollination, the progression of the pollen tube may bring the sperm cells into the female gametophyte before the completion of a cell wall at the chalazal region. While some authors suggested that a cell wall might impede fertilization of the apomictic egg cell (Asker and Jerling, 1992; Savidan, 1992), no cytological evidence has been given to support these hypotheses. Recent findings which show that the

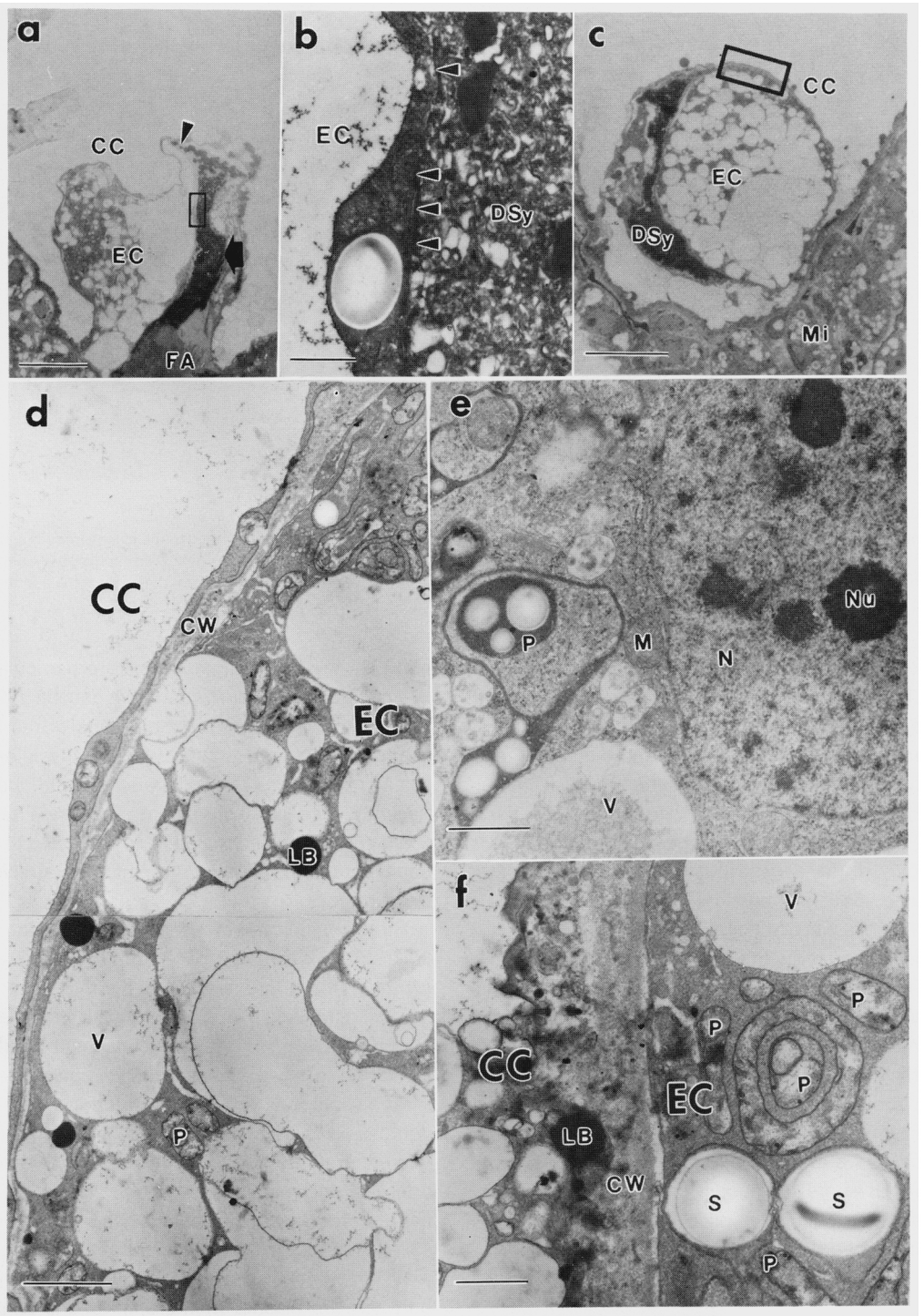

Figure 4. The egg apparatus of *P. ciliare* 4 hours after pollination; sexual egg apparatus (a and b) and aposporous egg apparatus (c–f).
(a) In a sexual female gametophyte, a synergid has degenerated (arrow), and a fringe of collapsed cytoplasm (arrowhead) has invaded the space between the egg cell (EC) and the central cell (CC). The region outlined is depicted in (b); FA, filiform apparatus; bar=10 μm.
(b) Detail of (a). No cell wall is present at the chalazal region of the egg cell, and the plasma membrane of the degenerated synergid (DSy) and the egg cell (EC) appear to be in contact (arrowheads); bar=1 μm.
(c) In an aposporous female gametophyte, a degenerated synergid (DSy) appears in association with a highly vacuolated egg cell (EC). The region outlined is depicted in (d); CC, central cell; Mi, micropyle; bar=10 μm.
(d) Detail of (c). The chalazal region of the aposporous egg cell (EC) is covered by a cell wall (CW); its cytoplasm appears vacuolated and contains undifferentiated plastids (P). A thin layer of cytoplasm in the central cell (CC) is associated with the external side of the egg cell wall. V, vacuole; LB, lipid body; bar=2 μm.
(e) In the cytoplasm of the egg cell, the nucleus (N) is often associated with numerous plastids (P) and mitochondria (M). V, vacuole; Nu, nucleolus; bar=1 μm.
(f) Association of undifferentiated plastids (P) in the cytoplasm of the aposporous egg cell (EC); CW, cell wall; LB, lipid body; CC, central cell; P, plastid; S, starch; V, vacuole; bar=1 μm.

frequency of fertilization of apomictic egg cells can be increased through early pollination (Martinez *et al.*, 1994) are consistent with the evidence from this study.

The most fundamental problems related to double fertilization in higher plants are far from being solved. Their elucidation will depend on taking advantage of the reproductive diversity inherent in angiosperms; the members of the Poaceae are particularly attractive in this regard. This study suggests that the examination of apomictic reproductive characteristics represents a valuable method to obtain information on specific cellular aspects of double fertilization in flowering plants.

Experimental procedures

Plant material

Plants were grown in individual pots in the greenhouse facilities (T=15°–30°C; RH=60–90%) of the Department of Soil and Crop Sciences (Texas A&M University). For each genotype (BBW178, BBW291, and T4464), whole tillers with immature inflorescences (90–100% of the inflorescence out of the flag leaf and no stigma extrusion) were removed from the plant, placed in distilled water, and cross-pollinated under standard laboratory conditions (T=25°C; 12 h of light).

Light microscopy observation of cleared pistils

Unpollinated mature florets from isolated inflorescences were dissected and fixed in FAA for 12 h at 25°C. Florets were allowed to sink by placing the vials containing the specimens in vaccum for 30 min. After fixation, pistils were dissected, and stored in 70% ethanol (in double-distilled H_2O). Subsequently, the material was cleared in methyl salicylate according to Young *et al.* (1979), and whole-cleared pistils were sagittally mounted in the same clearing media. Cytological analysis was conducted using phase-contrast and Nomarsky interference optics (Axioskop[a], Zeiss, Germany). Micrographs were obtained using Tmax 100, Tech Pan 100, and Ektar 100 film (Kodak[a], USA).

Observation of sectioned material.

One day before anthesis, mature isolated inflorescences with fully extruded stigmas were cross-pollinated. In *Pennisetum ciliare*, fertilization occurs 4–6 hours after pollination (Bashaw, personal communication). Unpollinated and pollinated pistils were fixed 0–4 h after pollination at 1/2 h intervals. Pistils dissected at different hs after pollination were submerged in a drop of fixative, and the style and the upper chalazal region of the ovary were carefully trimmed with a razor blade to enhance fixative penetration (Chaubal and Reger, 1992). Fixation was in 3% glutaraldehyde in 50 mM cacodylate buffer (pH=7.2) for 2 h at room temperature (T=25°C). Following three rinses in the same buffer (10 min each), samples were postfixed in 2% OsO_4 in cacodylate buffer for 2 h at room temperature, dehydrated in an ethanol series (10% steps) and flat embedded in Spurr's low-viscosity resin (Spurr, 1969). Ovaries in the polymerized resin were reoriented by mounting previously flat-embedded specimens on blank resin pyramidal blocks. Semi-thin (0.5–1 μm) and thin sections (0.07–0.09 μm) were cut with an ultramicrotome (Ultracut E[a], Reichert-Jung, Austria) using glass or diamond knives and collected on hexagonal-mesh nickel grids, or collodion-coated, slotted copper grids.

Semi-thin sections were stained with 1% toluidine blue in sodium metaborate buffer. To verify the absence of pollen tube entry into the female gametophyte, ovules selected for ultrastructural observations were completely sectioned after collection of thin sections. In pollinated specimens, tests for localization of carbohydrate spherical particles within the synergids were conducted using the periodic acid-Schiff (PAS) procedure described by Jensen (1962). Semi-thin sections were observed and photographed under conventional brightfield and phase contrast optics using Tmax 100 or Tech Pan 100 films (Kodak[a], USA). Some semi-thin sections were re-embedded using the procedure described by Kennell (1984). Thin sections from this re-embedded material or from consecutive sagittal sections were poststained with uranyl acetate and Reynold's lead citrate, and examined using a transmission electron microscope (Zeiss 10C[a], Germany) at 60 or 80 kV.

[a]Mention of a trade mark of a propietary product does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of the products that may also be suitable.

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References

Asker, S.E. and Jerling, L. (1992) *Apomixis in Plants.* Boca Raton, FL: CRC Press.

Bashaw, E.C. and Hanna, W.W. (1990) Apomictic reproduction. In *Reproductive Versatility in the Grasses* (Chapman G., ed.). Cambridge: Cambridge University Press, pp. 100–130.

Bashaw, E.C. and Hignight, K.W. (1990) Gene transfer in apomictic buffelgrass through fertilization of an unreduced egg. *Crop Sci.* **30**, 571–575.

Bogdan, A.V. (1977) *Tropical Pasture and Fodder Plants.* Longman, London.

Chaubal, R. and Reger, B.J. (1992) Calcium in the synergid cells and other regions of pearl millet ovaries. *Sex. Plant Reprod.* **5**, 34–46.

Diboll, A.G. (1968) Fine structural development of the megagametophyte of *Zea mays* following fertilization. *Am. J. Bot.* **55**, 787–806.

Dickinson, H.G. (1992) Plant reproduction: past, present and future. In *Reproductive Biology and Plant Breeding* (Dattée, Y., Dumas, C. and Gallais, A., eds). Berlin: Springer-Verlag, pp. 15–27.

Friedman, W.E. (1990) Double fertilization in *Ephedra,* a nonflowering seed plant: its bearing on the origin of angiosperms. *Science,* **247**, 951–954.

Friedman, W.E. (1991) Double fertilization in *Ephedra trifurca,* a non-flowering seed plant: the relationship between fertilization events and the cell cycle. *Protoplasma,* **165**, 106–120.

Gustafsson, Å. (1947) Apomixis in angiosperms II. *Lunds Univ. Årsskr N F II*, **43**, 71–179.

Harlan, J.R. and de Wet, J.M.J. (1963) Role of apomixis in the

evolution of the *Botrichloa-Dichanthium* complex. *Crop Sci.* **3**, 314–316.

Huang, B.Q. and Russell, S.D. (1992) The female germ unit. In *Sexual Reproduction in Flowering Plants* (Russell, S.D. and Dumas, C., guest eds). *Int. Rev. Cytol.* **140**, 233–293.

Hussey, M.A., Bashaw, E.C., Hignight, K.W. and Dahmer, M.L. (1991) Influence of photoperiod on the frequency of sexual embryo sacs in facultative apomictic buffelgrass. *Euphytica,* **54**, 141–145.

Jensen, W.A. (1962) *Botanical Histochemistry.* San Francisco: W.H. Freeman and Co.

Jensen, W.A. and Fisher, D.B. (1968) Cotton embryogenesis: the entrance and discharge of the pollen tube in the embryo sac. *Planta,* **78**, 158–183.

Kennell, J.C. (1984) Influence of the soybean male-sterile gene (ms1) on the development of the female gametophyte. *M Sc Thesis.* Ames, IA: Department of Botany, Iowa State University.

Knox, R.B. (1967) Apomixis: seasonal and population differences in a grass. *Science,* **157**, 325–326.

Martinez, E.J., Espinoza, F. and Quarin, C.L. (1994). BIII progeny (2n+n) from apomictic *Paspalum notatum* obtained through early pollination. *J. Hered.* **85**, 295–297.

Mogensen, H.L. (1982) Double fertilization in barley and the cytological explanation for haploid embryo formation, embryoless caryopses, and ovule abortion. *Carlsberg Res. Commun.* **47**, 313–354.

Mogensen, H.L. (1988) Exclusion of male mitochondria and plastids during syngamy in barley as a basis for maternal inheritance. *Proc. Natl Acad. Sci. USA,* **85**, 2594–2597.

Mòl, R., Matthys-Rochon, E. and Dumas, C. (1994) The kinetics of cytological events during double fertilization in *Zea mays* L. *Plant J.* **5**, 197–206.

Nogler, G.A. (1984) Gametophytic apomixis. In *Embryology of Angiosperms* (Johri, B.M., ed.). New York: Springer-Verlag, pp. 475–518.

Russell, S.D. (1992) Double fertilization. In *Sexual Reproduction in Flowering Plants* (Russell, S.D. and Dumas, C., guest eds). *Int. Rev. Cytol.* **140**, 357–388.

Savidan, Y.H. (1992) Progress in research on apomixis and its transfer to major grain crops. In *Reproductive Biology and Plant Breeding* (Dattée, Y., Dumas, C. and Gallais, A., eds). Berlin: Springer-Verlag, pp. 269–279.

Schulz, S.R. and Jensen, W.A. (1968) *Capsella* embryogenesis: the egg, zygote and young embryo. *Am. J. Bot.* **55**, 807–819.

Sherwood, R.T., Young, B.A. and Bashaw, E.C. (1980) Facultative apomixis in buffelgrass. *Crop Sci.* **20**, 375–379.

Spurr, A.R. (1969) A new viscosity resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**, 31–43.

Taliaferro, C.M. and Bashaw, E.C. (1966) Inheritance and control of obligate apomixis in breeding buffelgrass, *Pennisetum ciliare. Crop Sci.* **6**, 473–476.

Young, B.A., Sherwood, R.T. and Bashaw, E.C. (1979) Cleared pistil and thick sectioning techniques for detecting aposporous apomixis in grasses. *Can. J. Bot.* **57**, 1668–1672.

Zaki, M.A.M. and Dickinson, H.G. (1990) Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus. Protoplasma,* **156**, 149–162.